

Atty Dkt. No.: UCAL142CON  
USSN: 08/976,560  
Exhibit 1

| CERTIFICATE OF MAILING  |   |   |
|---|---|---|
| I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231. |   |   |
| Typed or Printed Name   | <i>Cindy Hoang</i>                        |   |
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| <b>DECLARATION OF<br/>L. ALISON MCINNES<br/>UNDER 37 C.F.R. § 1.132</b><br><br>Address to:<br>Assistant Commissioner for Patents<br>Washington, D.C. 20231  | Attorney Docket Confirmation No.          | UCAL142CON<br>2046  |
|   | First Named Inventor                      | N.B. Freimer  |
|   | Application Number                        | 08/976,560  |
|   | Filing Date                               | November 24, 1997   |
|   | Group Art Unit                            | 1655  |
|   | Examiner Name                             | L. Arthur   |
|   | Title                                     | <i>Methods for treating bipolar mood disorder associated with markers on chromosome 18p</i> |

Dear Sir:

1. I, L. Alison McInnes, declare and say I am a co-inventor of the claims of the above-identified patent application. I directed others and personally performed the research leading to the invention disclosed and claimed therein.

2. I have read the Office Action dated April 24, 2001 in this application and understand that the Examiner has rejected pending claims 1-12 and 25-27 on the basis that the specification is not enabling for the full scope of the claims.

3. The data presented below show that, using techniques described in the specification, at least five new polymorphisms, including single nucleotide polymorphisms (SNP), were identified in the narrow interval on chromosome 18p described in the application, which polymorphisms are associated with bipolar mood disorder (BP). Thus, in addition to the polymorphisms already identified in the patent application, and using the guidance provided in the application, several additional polymorphisms were identified that are associated with BP.

**ASSOCIATION OF POLYMORPHISMS WITH BP IN A NARROW INTERVAL ON CHROMOSOME 18P  
AS IDENTIFIED IN THE INSTANT APPLICATION AND CORROBORATED BY SUBSEQUENT WORK**

4. The instant application provided data showing a positive LOD score for a D18S59 allele with BP in a pedigree analysis; and gave evidence of an association of D18S59 with BP in a population study. The instant application further showed a positive LOD score for a D18S476 allele with BP in a pedigree analysis and gave evidence of association in population studies. In a subsequent study of linkage disequilibrium (LD) on chromosome 18 in a population sample of 69 BP-I patients from the Central Valley of Costa Rica (CVCR), the same D18S59 allele was associated with BP-I. Escamilla et al. (1999) *Am. J. Hum. Genet.* 64:1670-1678; a copy of which is provided herewith as Exhibit 2.

5. Further genotyping of the 69 affected individuals using four publicly available microsatellite markers delineated a segment of maximal LD with BP-I, covering about 331 Kb. Evaluation of a larger sample (227 patients and relatives, and 26 independent control trios) using these markers showed continuing evidence of LD and haplotype sharing in this sample for this region. Escamilla et al. (2001) *Am. J. Med. Genet.* 105:207-213; a copy of which provided herewith as Exhibit 3.

6. Thus, the instant application provides evidence of association of at least two polymorphisms associated with BP. This association was corroborated by work published after the filing date of the instant application. These markers are in a narrow interval between SAVA5 and ga203 on chromosome 18p. Within this region, a segment of about 331 kb, and having maximal LD with BP, was further delineated.

**AT LEAST FIVE ADDITIONAL POLYMORPHISMS ASSOCIATED WITH BP WERE FOUND IN THE  
PREVIOUSLY IDENTIFIED NARROW INTERVAL**

7. Using techniques described in the instant application, at least five additional polymorphisms were identified that are located within the narrow interval between SAVA5 and ga203, and that are associated with BP.

8. As described in detail below, four new microsatellite markers, and 26 new single nucleotide polymorphisms (SNPs) were identified in the narrow interval on chromosome 18p. The results of LD analysis of these 30 new markers, as well as four previously identified microsatellite markers, are displayed in Table 1. Of

the 34 markers presented in Table 1, 16 showed association ( $\lambda > 0$ ) with BP in at least one of the two samples. The p-value for five of these 16 markers was  $< 0.01$ . All five of these markers (PH84, PH205, PH202, PH208, and TS30) had estimates of  $\lambda$  near 1.0, indicating that virtually all affected individuals had at least one copy of the associated allele.

## **METHODS**

### **Sample collection**

9. Two samples were analyzed. In one sample, the patient sample was composed of 227 CVCR BP-I individuals (including the set of 69 patients from Escamilla (2001) that gave the original association evidence in 18p) and their available first degree relatives (total N=563). All affected individuals had at least two psychiatric hospitalizations with the first hospitalization by age 50. A second sample was comprised of these 563 individuals and a set of controls (52 unrelated parents of students recruited from the University of Costa Rica who were selected for CVCR ancestry [at least 5 out of 8 great-grandparents from the CVCR]).

### **Radiation hybrid and STS-content mapping of markers within the candidate interval**

10. Genetic and physical mapping information was initially obtained from Whitehead Institute for Biomedical Research/MIT Center for Genome Research, Stanford Human Genome Center, GÉNÉTHON Human Genome Research Center, and the Cooperative Human Linkage Center. Radiation hybrid (RH) mapping was used extensively in the early phase of this study to resolve discrepancies in marker order between maps. Specifically, the 83 Stanford G3 radiation hybrid panel was used to map all genetic and STS markers available from public database as well as those developed specifically for the project. In addition to RH mapping, STS-content mapping using BAC (Bacterial Artificial Chromosome) clones from the region of interest was also used routinely to determine the marker order and to complete the BAC contig.

### **BAC library screening, end sequencing and contig building**

11. Microsatellite and STS markers obtained from public databases were used to screen the human BAC library from Research Genetics (Huntsville, AL) by PCR or to the BAC library from Genome Systems (St. Louis, MO) screen by hybridization according to manufacturers' protocols. BAC DNA from positive clones was prepared, and sequences of the BAC ends were obtained by cycle sequencing the BAC DNA directly with vector primers T7 and SP6, respectively. PCR primers were designed from non-repetitive end sequences and used as STS markers to improve the physical map and the BAC contig construction. The

outlying markers from each side of the contigs were used to screen for overlapping BAC clones to extend the contigs.

#### **Construction of randomly sheared libraries from BACs**

12. BAC DNA was sheared to small fragments of desired size range using a nebulizer. After shearing, the libraries were constructed using established techniques.

#### **Microsatellite and SNP marker development and genotyping**

13. Microsatellite markers were generated by hybridizing oligonucleotide probes for di, tri, and tetranucleotide repeats to randomly sheared sub-libraries made from BAC clones using Quicklite non-isotopic enzyme induced chemiluminescent reagents from Lifecodes Corp. (Stamford CT) following the manufacturer's instructions. Positive clones were sequenced to identify microsatellite sequences and primers were then designed from flanking unique DNA sequence. Primers for amplifying STS markers were also designed using BAC end sequences, and random sequences available within the candidate interval when extensive sequencing of the randomly sheared libraries were done. Primer sequences are publicly available at PNAS Online.

14. We genotyped the 4 new microsatellites identified by us in sequencing the region. Primer sequences are available on request. Genotyping procedures for the microsatellites were performed using established techniques.

15. Single nucleotide polymorphisms (SNPs) were identified using SSCP (Single Strand Conformational Polymorphism) analysis of STS markers (all < 300 basepairs in length), using established techniques. We used four unrelated individuals to screen for each SNP. We genotyped the SNPs in patient and control samples using standard SSCP procedures.

#### **Sequencing of the candidate interval and identification of the candidate genes**

16. In the interval of < 3 cM, located within the SAVA5-ga203 interval, randomly sheared libraries prepared from BACs covering this region were sequenced at 10X coverage to discover all sequence information and identify all genes within the interval. More than 10,000 individual sequences from the region were compared by BLAST20 with sequences from publicly available databases and were analyzed using GRAIL21 to identify potential coding sequences. In addition, sequences were assembled using PHRAP 22,

23, 24 in a single DNA strand of ~331 Kb. The whole sequence was again analyzed using BLAST and GRAIL to aid in gene prediction. These data were displayed in ACEDb (data available from [ncbi.nlm.nih.gov](http://ncbi.nlm.nih.gov)) to visualize predicted exons and their relationships to each other.

### Statistical analyses

17. We applied a modified version of Terwilliger's likelihood ratio test of LD to the 4 novel microsatellites and 26 SNPs that spanned our 331 Kb candidate region. For each of these 30 markers we applied this test twice, once in the sample of 227 patients and their available relatives, and also with the addition of the independent controls to the 227 patients and relatives. This likelihood ratio test estimates a single parameter,  $\lambda$ , which quantifies the overrepresentation of an associated marker allele on disease chromosomes versus control chromosomes.  $\lambda$  is related to the common epidemiological parameter of population attributable risk. If the frequency of an associated allele on disease and normal chromosomes is given by  $p_D$  and  $p_N$ , respectively, then  $\lambda$  is calculated by  $(p_D - p_N)/(1 - p_N)$ . Only positive associations with disease are permitted, and  $\lambda$  ranges from 0 (under the null of no association) to 1.0 (all disease chromosomes carry the associated allele). Others have shown that  $\lambda$  is the most closely related to the recombination fraction with disease and less influenced by marker allele frequencies than other measures of LD. Because we do not know which chromosome of an affected individual harbors the disease locus, we incorporated a genetic model of disease transmission in the procedure of Terwilliger. Using this model also enabled us to employ data from additional family members other than parents, if they were not available. The same genetic model (mostly dominant with reduced penetrance) was used as in our previous LD papers and in the genome screen of the Costa Rican pedigrees described in McInnes et al. In this model one chromosome of the affected individual is used as a control chromosome. The use of a model is likely to increase the power of the test and the precision of the estimates of  $\lambda$  when the inheritance pattern is approximately known. Using simulated data, Terwilliger shows that his test is conservative.

## RESULTS

### Marker development and physical map

18. Based on our previous results (as described in the instant patent application; and in the publications provided herewith) we focused marker development and physical mapping efforts (including direct sequencing) in the <3 cM region between sAVA5 and D18S1231. Within this region we identified 4 new microsatellite markers and 26 SNPs to add to the 4 publicly available microsatellite markers already used

(see Exhibit 3). Based on the extent of haplotype sharing in pedigree CR001 and LD results from the previously used markers, we focused our detailed investigation on the region of about 331 Kb between PH33 and D18S1231 (although in public databases this segment is estimated as being 378 Kb in length, contig NT\_011005). Using several sequence analysis tools and database mining procedures (see Methods, above), we determined that this interval contained six known genes (*CENTRIN*, *CLUL1*, *TYMS*, *rTS*, *YES1*, and *ADCYAP1*, ordered from telomeric to centromeric, with *TYMS* and *rTS* overlapping each other). This order differs in the public database (*CENTRIN*, *CLUL1*, *YES1*, *rTS*, *TYMS*, and *ADCYAP1*, with no overlap between *rTS* and *TYMS*). All of the genes except "clusterin-like 1 (retinal)" gene [*CLUL1*] have been well characterized previously. *CLUL1* was originally identified during a screen of a human retinal cDNA library for retina-specific genes. The function of this gene is not known; however Northern blot analysis reveals that it is highly expressed in retina with much lower yet detectable expression in several other tissues including brain, kidney and testes.

#### Genotyping results

19. We genotyped the 30 new markers in pedigree CR001 and in the CVCR patient and control samples. Results of the LD analysis for these markers (and the four previously available markers reported in ref 8) are displayed in Table 1 (provided herewith as Exhibit 4). Of the 34 markers presented in Table 1, 16 showed association ( $\lambda > 0$ ) with BP-I in at least one of the two samples (that with 227 patients/relatives and that with 227 patients/relatives and the addition of 52 controls). The p-value associated with the estimate of  $\lambda$  was  $< 0.01$  for five of these 16 markers, and for four of the five markers the magnitude of association was greater in the sample containing the population controls. All five of these markers (PH84, PH205, PH202, PH208, and TS30), had estimates of  $\lambda$  near 1.0, indicating that virtually all affected individuals had at least one copy of the associated allele. The markers showing LD are clustered in the 19 Kb segment between exon 8 of *CLUL1* and exon 1 of *TYMS*. This segment also contains the minimal region of haplotype sharing within CR001, and for each marker in this segment, the associated alleles seen in the population samples are the same alleles in the shared haplotype in CR001 (last column in Table 1).

**SUMMARY**

20. The data presented herein extend the findings described in the instant patent application. The patent application provided evidence, from both pedigree analyses and population studies, that a number of polymorphisms, including a 154 bp allele of the microsatellite marker D18S59 and a 271 bp allele of the microsatellite marker D18S476, are associated with BP. The patent application described how to identify additional markers, and how to determine whether such markers are associated with BP.

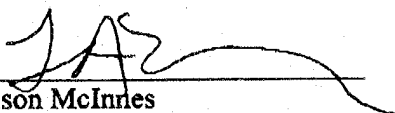
21. The data presented herein show that, using techniques described in the patent application, several new polymorphisms, located in the previously identified interval and associated with BP, were identified.

**CONCLUSION**

22. Those in the field, given the guidance in the instant patent application, could identify additional polymorphisms associated with BP.

23. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code, and that such will false statements may jeopardize the validity of the application or any patent issuing thereon.

Oct 9 2001  
Date

  
L. Alison McInnes

Enclosures: Exhibits 2-4



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Exhibit 2 of 4  
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# Assessing the Feasibility of Linkage Disequilibrium Methods for Mapping Complex Traits: An Initial Screen for Bipolar Disorder Loci on Chromosome 18

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## Summary

Linkage disequilibrium (LD) analysis has been promoted as a method of mapping disease genes, particularly in isolated populations, but has not yet been used for genome-screening studies of complex disorders. We present results of a study to investigate the feasibility of LD methods for genome screening using a sample of individuals affected with severe bipolar mood disorder (BP-I), from an isolated population of the Costa Rican central valley. Forty-eight patients with BP-I were genotyped for markers spaced at ~6-cM intervals across chromosome 18. Chromosome 18 was chosen because a previous genome-screening linkage study of two Costa Rican families had suggested a BP-I locus on this chromosome. Results of the current study suggest that LD methods will be useful for mapping BP-I in a larger sample. The results also support previously reported possible localizations (obtained from a separate collection of patients) of BP-I-susceptibility genes at two distinct sites on this chromosome. Current limitations of LD screening for identifying loci for complex traits are discussed, and recommendations are made for future research with these methods.

## Introduction

Identifying genes for disorders with complex inheritance patterns is one of the greatest challenges in biomedical research (Lander and Schork 1994). Such disorders, which include many of the most prevalent human diseases, are difficult to map with standard linkage methods. It has been suggested that the availability of dense marker maps covering the genome will make linkage disequilibrium (LD) analysis a feasible approach for screening the genome to map complex disorders (Risch and Merikangas 1996). Current marker maps are not sufficiently dense to enable such studies to be performed in heterogeneous populations or in populations that were founded in the distant past. However, the success of genome-screening LD-mapping studies of genetically simple and/or rare diseases in recently founded isolated populations (Houwen et al. 1994; Puffenberger et al. 1994; Friedman et al. 1995; Newport et al. 1996) provide the impetus for testing the utility of LD methods for mapping complex diseases in such populations (Escamilla et al. 1996). In populations where randomly sampled patients are on average <20 generations removed from their last common ancestor, LD may be maintained for sizable regions around disease genes. Such LD should be manifested by affected individuals sharing alleles, identical by descent (IBD), at markers spaced at intervals of several centimorgans surrounding

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<sup>§</sup>Dr. Gallegos died after this paper was accepted for publication. This paper is dedicated to his memory.

a disease gene. We now present the results from the first stage of a study in which LD methods were used to screen for loci that predispose to severe bipolar mood disorder (BP-I), which is common and is almost certainly characterized by a complex mode of inheritance. The study was done in a relatively recently founded isolated population, that of the central valley of Costa Rica (CVCr) (Escamilla et al. 1996), where founder effects have already been observed for several inherited diseases (Saborio 1992; Uhrhammer et al. 1995; Shah et al. 1997).

Despite long-standing evidence that BP-I has a genetic basis (Escamilla et al. 1997), genome scans for linkage have provided equivocal results (Risch and Botstein 1996; Nurnberger et al. 1997) that fail to satisfy the levels of significance suggested for genomewide screens by Lander and Kruglyak (1995). The failure to identify BP-I loci definitively, by standard linkage approaches, probably reflects uncertainty regarding mode of inheritance, high phenocopy rates, difficulty in demarcation of distinct phenotypes, and presumed genetic heterogeneity. LD-based mapping approaches within population isolates may offer a means of diminishing several of these obstacles. An approach (such as LD mapping) that samples individuals from an entire population can more easily ascertain a large set of patients with a narrowly defined, reliably diagnosed phenotype (in this case, BP-I) than linkage-based approaches that require ascertainment of family units with multiple affected cases. Within a population isolate, genetic heterogeneity of BP-I may also be less than in larger, genetically mixed populations, as there is a high probability that individuals with such a phenotype share descent from a few common ancestors.

We collected a sample of patients with BP-I, for LD analysis, by identifying individuals currently living in the CVCr who had known CVCr ancestry. This sample was collected independently of our previous pedigree-based studies of BP-I in Costa Rica. Our aim in the current study was to evaluate the feasibility of identifying BP-I loci by LD screening in this population, as proposed in Escamilla et al. (1996). To do this, we conducted an LD screen of an entire single chromosome (chromosome 18). This chromosome was chosen because previous linkage studies in Costa Rica and in other populations suggested that it possibly contained bipolar disorder loci (Berretini et al. 1994; Stine et al. 1995; Freimer et al. 1996a). Genealogical studies indicated that the individuals in our current study did not share common ancestry over the past several generations (Escamilla et al. 1996). We therefore anticipated that we would not detect random genome regions shared IBD by more than a few individuals and that regions of high IBD sharing would thus be areas containing possible BP-I-susceptibility genes inherited from a common founder.

## Samples and Methods

### Sample Collection

To diminish the likelihood of investigating phenocopies, we limited the sample to individuals with a definite diagnosis of BP-I, with onset by age 50 years and a history of at least two psychiatric hospitalizations. The 48 patients with BP-I (25 female patients and 23 male patients) in the current study were recruited independently from psychiatric hospitals and clinics in the CVCr. First-degree relatives of patients were also recruited, to determine genetic phase. The study was approved by institutional review boards at the Costa Rican Ministry of Health, the University of Costa Rica, and the University of California at San Francisco, and informed consent was obtained from all participating subjects. Of the 48 BP-I subjects, 8 individuals had both parents available for genotyping, 20 individuals had one parent available, 10 individuals had one or more children available, 1 individual had two siblings available, and 9 individuals had no relatives available. In nuclear families, only one individual (the proband) was designated as affected, and all others were considered to have unknown phenotype. Details of ascertainment and diagnostic procedures, and the clinical and genealogic profiles of the study sample, can be found in Escamilla et al. (1996).

### Genotyping

We used 26 markers, spanning chromosome 18, to genotype all 48 affected individuals (as well as 53 relatives, to establish phase). Of the 25 regions, 21 were  $\leq 6$  cM, and 4 were 6–7 cM. The average distance between markers was 4.8 cM. When choices were available, we chose the most polymorphic marker (Gyapay et al. 1994). The average heterozygosity of the markers used in this screen (in the CEPH pedigree collection) was 0.75. (The only screening markers with heterozygosity values  $< 0.70$  were D18S464, D18S60, D18S378, and D18S469.) We screened chromosome 18 at a marker density of 6 cM because available marker maps had gaps  $\leq 6$  cM, and our goal was to have an equal density of coverage across the chromosome (Gyapay et al. 1994). We chose markers from the maps available, at the time of the study, from Génethon (Gyapay et al. 1994), the Cooperative Human Linkage Center (Murray et al. 1994), and the public database of the Utah Center for Genome Research. Genotyping procedures used for all experiments were as previously described by Di Rienzo et al. (1994). In brief, one of the two primers was labeled radioactively with a polynucleotide kinase, and PCR products were separated, by electrophoresis, onto polyacrylamide gels. Autoradiographs were scored independently by two raters. Data for each marker were entered



Table 1

Heterozygosity of Markers Used in the Genome Screen of Chromosome 18

| Marker Name           | Heterozygosity in<br>Génethon Database | Heterozygosity in<br>Costa Rican Sample <sup>a</sup> |
|-----------------------|--|--|
| D18S1140              | .49                                    | .39  |
| D18S59 <sup>b</sup>   | .81                                    | .81  |
| D18S476 <sup>b</sup>  | .76                                    | .62  |
| D18S481               | .76                                    | .74  |
| D18S391               | .75                                    | .69  |
| D18S452               | .83                                    | .85  |
| D18S843               | NA                                     | .73  |
| D18S464               | .65                                    | .51  |
| D18S1153              | .78                                    | .69  |
| D18S378               | NA                                     | .54  |
| D18S53                | .79                                    | .81  |
| D18S453               | .82                                    | .81  |
| D18S40                | NA                                     | .81  |
| D18S66                | .85                                    | .81  |
| D18S56                | .73                                    | .74  |
| D18S57                | .87                                    | .85  |
| D18S467 <sup>b</sup>  | .73                                    | .64  |
| D18S460               | .62                                    | .67  |
| D18S450               | .79                                    | .74  |
| D18S474               | .82                                    | .73  |
| D18S69                | .79                                    | .78  |
| D18S64                | .74                                    | .65  |
| D18S1134              | .73                                    | .68  |
| D18S1147              | .85                                    | .86  |
| D18S60                | .37                                    | .58  |
| D18S55                | .77                                    | .80  |
| D18S68                | .79                                    | .79  |
| D18S477               | .62                                    | .70  |
| D18S61 <sup>b</sup>   | .87                                    | .86  |
| D18S488               | .87                                    | .82  |
| D18S485 <sup>b</sup>  | .79                                    | .79  |
| D18S541               | NA                                     | .63  |
| D18S870 <sup>b</sup>  | NA                                     | .66  |
| D18S469 <sup>b</sup>  | .65                                    | .64  |
| D18S874               | NA                                     | .64  |
| D18S380               | NA                                     | .63  |
| D18S1121 <sup>b</sup> | .74                                    | .77  |
| D18S1009              | .74                                    | .66  |
| D18S844               | NA                                     | .76  |
| D18S554               | .82                                    | .79  |
| D18S461               | .77                                    | .65  |
| D18S70                | .83                                    | .86  |

NOTE.—NA = data not available.

<sup>a</sup> Allele frequencies were calculated from the entire sample, accounting for known relationships among individuals.<sup>b</sup> Markers with  $-2\ln(LR) > 1.0$ .

nondisease chromosomes (Terwilliger 1995; Freimer et al. 1996a). A single parameter,  $\lambda$ , is estimated, which quantifies such overrepresentation of marker alleles on disease chromosomes. Designation of chromosomes of probands as disease carrying or non-disease carrying was achieved by specification of a genetic model for the disease. The same model of transmission was used in this LD-likelihood test as was used in the initial genome screen of the Costa Rican families, described in McInnes et al. (1996). In brief, this model assumes that the disease

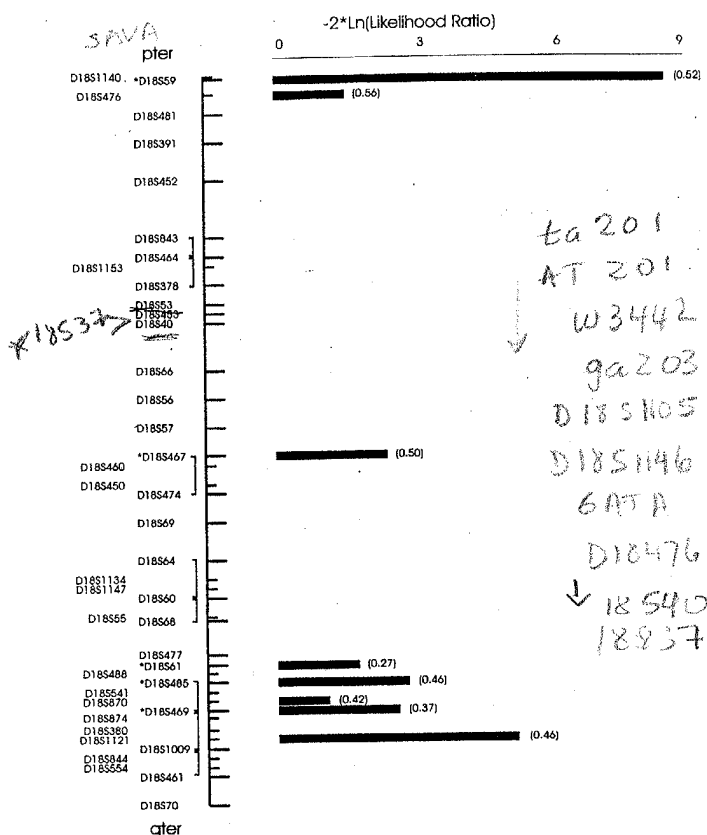
is nearly dominant (assuming penetrance of .81 for heterozygous individuals and .90 for homozygous individuals with the disease mutation), that the population prevalence of BP-I in Costa Rica is .015, and that the frequency of the disease gene in the population is .003. In the likelihood calculations, all possible disease-genotype combinations of all relatives are considered. With the model that was used, in which the disease-gene frequency is very low, the LD-likelihood test, in most cases, treats the probands as effectively heterozygous at the disease locus, and chromosomes of other relatives not occurring in the probands are treated as non-disease-carrying chromosomes. We did not specify a phenocopy rate in the genetic model, because the effect of phenocopies will be absorbed by the parameter  $\lambda$ ; the presence of phenocopies in our sample will serve to erode the association between marker alleles and disease and hence will reduce the estimate of  $\lambda$ . Because, in the present LD study, we were attempting to gather further evidence regarding the findings published in our initial genome screen, we limited ourselves to this one model in performing the likelihood analyses. However, both the BP-I family sample and the current LD sample will ultimately be analyzed with use of other models. We considered as promising those markers that gave evidence of overrepresentation of an allele on affected chromosomes, with a  $-2\ln(\text{likelihood ratio [LR]})$  statistic  $> 1.0$ .

Follow-up genotyping and LD-analysis studies were performed on markers that gave suggestive findings in the shared-segment evaluation. Within each segment that passed the threshold described above, 1–3 additional markers were typed to permit us to test for LD across regions of 1–2 cM. Markers that provided suggestive evidence of LD by the initial likelihood-ratio test, but had not been suggested as promising by the shared-segment screen, were also followed up, in this case by typing two additional nearby markers. In all, a total of 42 markers from chromosome 18 were used to genotype the study sample (table 1 and fig. 1). LD analysis of the additionally typed markers was conducted by use of the likelihood-ratio test.

## Results

### Simulations

Simulation results for the sample of 48 patients with BP-I and available relatives showed relatively high power to detect suggestions of association ( $P \leq .05$ ) with low phenocopy rates (94% for a phenocopy rate of 0%, and 54% for a phenocopy rate of 33%) but a dramatically decreased power under high phenocopy rates (e.g., 9% for a phenocopy rate of 67%). Additional simulations showed that, under higher phenocopy rates, the power to detect LD can be improved by increasing the sample



**Figure 1** Results from the LD screen of chromosome 18. The 26 markers used in the first stage of the screen are listed in the right column. Sixteen markers used to follow up interesting regions are listed in the left column. Approximate chromosomal locations of the 26 initial markers and the 16 follow-up markers are indicated by long and short tick marks, respectively. The eight segments that passed the initial screen threshold for segment sharing (50% of individuals or 25% of chromosomes sharing a two-marker haplotype) and the five markers that passed the initial threshold for the Terwilliger LR test ( $-2\ln[LR] > 1.0$ ) are indicated by blackened bars and asterisks, respectively. Two marker segments that passed the initial threshold were followed up by at least one marker within the segment, if possible (at the time of the study no markers were available between D18S843 and D18S464, and only one marker was available between D18S464 and D18S378). Markers that passed the initial threshold for the Terwilliger LR test were followed up with two additional markers. These additional markers flanked the original finding. The value of the  $-2\ln[LR]$  statistic, from the Terwilliger test, is plotted as a solid bar. This statistic is distributed as a one-sided  $\chi^2$  random variable with one degree of freedom. The estimate of the  $\lambda$  value, for the eight markers with positive results, is indicated in parentheses after the  $-2\ln[LR]$  statistic. Markers without a  $-2\ln[LR]$  statistic plotted had estimates of  $\lambda = 0$ , with the exception of three markers that had estimates of  $0 < \lambda < 0.62$ .

size and/or the marker density of screening (table 2). For instance, with a phenocopy rate of 75%, the power increases to 82% with a sample of 300 affected individuals and a 2.5-cM marker map.

### Shared-Segment Screen

We evaluated 25 possible shared segments (defined by the 26 markers genotyped in the sample). Eight regions passed the threshold of possible IBD sharing by  $\geq 50\%$  of patients. These regions were bounded by the following markers: D18S843-D18S464, D18S464-D18S378, D18S467-D18S474, D18S64-D18S60, D18S60-D18S68, D18S485-D18S469, D18S469-D18S1009, and D18S1009-D18S461 (fig. 1).

### Linkage-Disequilibrium Testing

Five (D18S59, D18S467, D18S61, D18S485, and D18S469) of the original 26 markers displayed evidence of possible LD, by means of a likelihood procedure ( $-2\ln[LR]$  statistic  $> 1.0$ ; table 3). Two (D18S59 and D18S61) of these five markers had not been identified as markers of interest by the shared-segment evaluation. D18S59, located near 18pter, displayed the strongest pointwise evidence for LD ( $-2\ln[LR]$  statistic of 8.3,  $P = .002$ ) of all the markers tested in this sample.

### Follow-up of Initial Results

Using the protocol discussed in Samples and Methods, we genotyped additional markers within the segments that passed the shared-segment screen as well as follow-up markers surrounding one (D18S59) of the two markers that had passed only the LD screen. We were unable to follow up one shared-segment region (D18S843-D18S464), because additional polymorphic markers were not available within the segment. We were also unable to follow up the finding for D18S61, for the same reason. Three (D18S476, D18S870, and D18S1121) of the 16 follow-up markers typed displayed additional evidence of possible LD (fig. 1).

These additional results brought to eight the total number of markers with  $-2\ln[LR]$  statistics  $> 1.0$  (table 3). Five of these eight marker loci were clustered within a small region of 18q22-23. The most significant LD in 18q22-23 was observed at D18S1121, with  $-2\ln[LR]$  of 5.03 and  $P = .01$ , and two were in 18pter.

For the two 18pter markers (D18S59 and D18S476),

**Table 2**

**Power-of-Likelihood-Analysis Test of LD**

| PENOCOPY RATE | POWER TO DETECT LD FOR<br>SAMPLE SIZE (N) = |     |      |      |
|---------------|---|-----|------|------|
|               | 90  | 200 | 300  | 400  |
| 50%           | 82%   | 99% | 100% | 100% |
| 75%           | 33%   | 62% | 82%  | 90%  |

NOTE.—Assumptions included that subjects were removed from a common ancestor by 10 generations, that a marker map of 2.5 cM was used, and that each marker had four equally frequent alleles. Values are the percentage of replicates to have  $P$  values  $< .05$ .

### Table

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Table 3

Frequencies of Marker Alleles Overrepresented in Disease Chromosomes, as Compared with Nondisease Chromosomes, for Markers Where  $-2\ln(LR) > 1.0$

| MARKER               | ALLELE | FREQUENCY ON           |                     |
|----------------------|--------|------------------------|---------------------|
|                      |        | Nondisease Chromosomes | Disease Chromosomes |
| D18S59 <sup>a</sup>  | 154    | .121                   | .572                |
| D18S476              | 271    | .470                   | .771                |
| D18S467 <sup>a</sup> | 172    | .384                   | .693                |
| D18S61 <sup>a</sup>  | 177    | .074                   | .326                |
| D18S485 <sup>a</sup> | 182    | .237                   | .586                |
| D18S870              | 179    | .405                   | .657                |
| D18S469 <sup>a</sup> | 234    | .128                   | .450                |
| D18S1121             | 168    | .171                   | .553                |

<sup>a</sup> Markers from the screening stage.

the alleles overrepresented on BP-I chromosomes (154 and 271 bp, respectively) form a haplotype that occurs in 48% of the patients with BP-I. Overall, this haplotype occurs on 26% of the chromosomes of individuals with BP-I and on 4% of the chromosomes not transmitted from parents to individuals with BP-I (definite phase for these two markers could be assigned in 25 patients with BP-I [50 chromosomes] and 25 nontransmitted parental chromosomes). Because the composite genetic and physical maps of the 18q22-23 region had not yet been completed at the time of this study, the relative order of the five markers in 18q22-23, for which evidence of LD was observed, was still too uncertain to permit construction of definitive marker haplotypes in our study sample.

Marker D18S467, in the 18q12.3 region, was the one marker outside 18q22-23 and 18pter to show a  $-2\ln(LR) > 1$  ( $-2\ln[LR] = 2.5$ ,  $P = .06$ ). The additional markers used to follow up this result (D18S450, D18S460, and D18S57) displayed no evidence of association.

#### Marker Heterozygosity in the Costa Rican Sample

We calculated heterozygosity values for the markers used, on the basis of the allele frequencies, estimated from the entire sample, accounting for known relationships among individuals. These heterozygosities are shown in table 1, along with the corresponding heterozygosity values of these markers in the CEPH population, used by Génethon.

#### Discussion

##### Screening for Complex Disease Loci by LD Approaches

Our intention in this work was to explore the feasibility of using LD methods to screen the genome for susceptibility genes for a common, genetically complex

disorder. The results obtained in our LD-based search for possible BP-I gene-loci on chromosome 18 were encouraging (specific susceptibility regions were suggested), but they highlight a number of issues that must be considered before LD screening is widely adopted.

Successful application of a shared-segment approach to any LD study depends on (1) a marker-map density that is appropriate to the age of the population isolate being studied and (2) a sharing threshold that will not be too high to allow true IBD areas to be identified and that will not be so low as to include many areas that are IBS false-positive signals. An appropriate marker map for an LD-screening study should have segments of a size expected to be shared IBD by many of the affected individuals. In addition to the density of the marker map used, the number of generations separating affected individuals from their common ancestor and the rate of etiologic heterogeneity in the population will also influence the choice of the sharing threshold, used to trigger further study. For example, if the common (disease-gene bearing) ancestor is removed from the current descendants by  $>10$  generations, the length of true IBD haplotypes shared by  $\geq 50\%$  of the descendants may be  $<5$  cM (and certainly  $<6$  cM, as is the screen used in this study) (Te Meerman et al. 1994; Durham and Feingold 1997). Our choice of a threshold of 50% of affected individuals sharing a possible haplotype therefore effectively meant that we were likely to identify only BP-I genes of a major effect in this population (phenocopy rate approaching zero), and even then, only if the distance from a common ancestor is not  $> \sim 10$  generations. Although this was probably too stringent a screen threshold, given the complex etiology of bipolar disorder, the alternative we faced—reducing the threshold to a lower percentage of potential IBD sharing—would have drastically decreased the specificity (and hence the utility) of the screen. For instance, in this particular study, lowering the threshold to a possible IBD haplotype shared in  $\geq 25\%$  of the patients would have resulted in 24 of the 25 regions tested being determined as regions of interest. If, in future studies, definite phase information can be set for a greater proportion of the probands (obtained from phasing information supplied by additional relatives) the “possible IBD” threshold will be more useful as a screening criterion at thresholds approaching 25% sharing (almost one in five of the patients with BP-I in the current study had no relative available for phase construction). Finally, regardless of the threshold chosen, there is no widely accepted statistical test available to evaluate the significance of the number of shared haplotypes observed, although several statistical approaches are under development (reviewed by Kruglyak 1997).

The use of markers with low heterozygosity will increase the number of false-positive results in a shared-

The third region that showed possible evidence of LD in our population sample was identified through a single marker (D18S467), in the 18q12.3 region. Additional markers typed near this one did not support the initial suggestion of LD in this region. Evidence from a linkage test that yielded a significance level of  $P = .06$  would be expected to occur, by chance, ~24 times (about once on most chromosomes) in a genomewide screen.

Our possible BP-I localizations at 18pter and 18q22-23, in the current sample, are distinct from regions on chromosome 18 suggested by other groups as being possibly linked to mood disorder (Berretini et al. 1994; Stine et al. 1995). We detected no evidence of association with these areas (near the centromere and in 18q21) in our BP-I population sample; nevertheless, the power of our current sample is not great enough to rule out these regions as potential BP-I loci in the Costa Rican population. McMahon et al. (1997) have recently reported excess allele sharing in sib pairs at 18S541, which is in the 18q22-23 region, although their affected status included not only BP-I, but also bipolar type II and schizoaffective patients.

#### *Future Directions*

The results of this study suggest that shared-segment-screening approaches will only be useful with the development of denser marker maps (Collins et al. 1997) and with the development of tests that permit statistical comparison of disease-chromosome haplotypes with control-chromosome haplotypes. Because the potential advantages of a shared-segment approach are substantial (this type of approach takes maximum advantage of the fact that haplotypes, not just single alleles, are inherited IBD in population isolates, and it is nonparametric), and because marker maps (Dib et al. 1996; Yuan et al. 1997) and statistical methods continue to improve, we remain optimistic about this method of mapping genes for complex disorders.

Both the 18pter and the 18q22-23 regions would have been identified as regions with possible LD at a significance of  $P < .05$ , even if we had not used the shared-segment approach but had instead screened for evidence by using only the likelihood-ratio test, with the original 26 markers. Our results indicate that, in population isolates, such as the CRCV, and with suitably dense marker coverage, tests similar to the likelihood-ratio test of LD (Terwilliger 1995) are promising tools for genome screening of complex diseases. It is not clear, however, whether currently available tests will be powerful enough to detect unequivocal proof of association in a genomewide scan for such diseases, given sample sizes that are easily obtained. More-powerful tests are needed and may emerge from efforts to develop measures that make use of haplotype information (Service et al. 1999

[in this issue]; Durham and Feingold 1997; Goldin and Chase 1997).

The test of LD screening conducted in the current study points out the need to do a more complete LD screening analysis. We thus intend to perform an LD screen of chromosome 18, using an expanded sample of patients with BP-I and a denser marker map. The addition of more-polymorphic markers to genome maps, and the application of haplotype-based statistical tests currently under development, should facilitate efforts to definitively identify BP-I susceptibility genes in Costa Rica.

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#### **Electronic-Database Information**

URLs for data in this article are as follows:

Cooperative Human Linkage Center, <http://www.chlc.org/> (for marker maps)  
G  n  thon, <http://www.genethon.fr/> (for marker maps)  
Utah Center for Genome Research, <http://www.genome.utah.edu/> (for marker maps)

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